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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:
HORI, ET AL.
Serial No. : 09/544,632
Filed: April 6, 2000
For: LIPID METABOLISM IMPROVING AGENT

Atty. Ref: 506.35379CC2
Group Art Unit: 1615
EXAMINER: Gollamudi S. Kishore, Ph. D

DECLARATION PURSUANT TO 37 C.F.R. 1.132

Sir:

I, Miho Takada of Niihari village, Ibaraki 500-4111, Japan hereby declare as follows,

I graduated from Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University in March, 1985. In April, 1987 I was employed by Kyowa Hakko Kogyo Co., Ltd. . In January, 1994 I transferred to Tsukuba research laboratories, and since then I have been engaged in research and development of functional food ingredients such as cholesterol-lowering materials.

The following experiments were conducted under my direction to examine the effect depending on the amount of bound phospholipid in the protein/phospholipid complex and the effect of binding protein with phospholipid to improve lipid metabolism in rats.

Experiment I

[Materials and methods]

Preparation of soy protein/phospholipid complex

Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was dispersed in water and then stirred at 10,000 rpm for 5 minutes to make a solution. Then phospholipid (SLP ; True lecithin, Mie, Japan) was added to the solution, and the mixture was stirred at 10,000 rpm for 5 minutes to prepare a solution containing soy protein/ phospholipid complex. The solution was freeze-dried to prepare three test samples having the ratios of soy protein to phospholipid being 9:1(ISP-10%PL), 4:1

(ISP-20%PL) and 1:1 (ISP-50%PL) , respectively.

The phospholipid was composed of the following (g/100g): phosphatidylcholine, 29.7; phosphatidylethanolamine, 23.3; phosphatidylinositol, 17.2; phosphatidic acid, 13.7; lysophosphatidylcholine, 3.7; phytic acid, 7.1.

Animals and diets

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) weighing about 110g were used. Room temperature was maintained at 22 ± 2°C with a 12 hour cycle of light (7:00-19:00) and darkness. All the rats were housed individually in metal cages and were allowed free access to diets and water. After acclimation to a commercial stock diet (CE-2; Japan CLEA, Tokyo) for 7 days, rats were divided into 4 groups of 6 rats on the basis of body weight. The rats of each group were given the diet comprising ISP, ISP-10%PL, ISP-20%PL or ISP-50%PL , respectively, as shown table 1, for 9 days. The composition of each diet was based on the American Institute of Nutrition (1977) formulation (AIN-76 diet) .

Table 1. Composition of the diets (g/kg)

	ISP	ISP-10%PL	ISP-20%PL	ISP-50%PL
Casein	166.1	166.1	166.1	166.1
ISP	58.8			
ISP-10%PL		65.4		
ISP-20%PL			73.5	
ISP-50%PL				117.6
Mineral mixture*	35	35	35	35
Vitamin mixture*	10	10	10	10
Choline chloride	2	2	2	2
Cholesterol	5	5	5	5
Sodium cholate	2.5	2.5	2.5	2.5
Lard	50	50	50	50
Corn oil	10	10	10	10
Sucrose	200	200	200	200
Cellulose	50	50	50	50
Starch	410.6	404	395.9	351.7

ISP, soyprotein ; PL, phospholipid ; ISP-10%PL, ISP-20%PL, ISP-50%PL, soyprotein with bound 10%, 20% and 50% phospholipids, respectively
* AIN 76 diet (American Institute of Nutrition, 1977)

The protein content of protein or each complex was as follows: soy protein(850g/kg), ISP-10%PL(765.0g/kg), ISP-20%PL(680.0g/kg), ISP-50%PL (425.0g/kg), casein (903.0g/kg). The lipid content of each protein was as follows: soy protein (15.0g/kg), ISP-10%PL (113.5g/kg), ISP-20%PL (212.0g/kg), ISP-50%PL (507.5g/kg).

Each rat was weighed its body weight and intake each 3days throughout this experiment with an electronic balance. At the end of this experiment ,after fasting for 18 hours, the rats were anesthetized with pentobarbital (50mg/kg B.W.). Blood was collected from abdominal artery of each rat. Whole liver was excised from each rat body. Each liver was weighed with an electronic balance after rinsed with ice-cold saline.

Lipid analysis

Serum was taken from blood with a centrifuge. Liver lipids were extracted with chloroform-methanol (2:1, v/v) in accordance with the Folch partition method, and total lipids were determined gravimetrically.

Lipid contents were determined by use of commercially available kits as follows: serum and liver cholesterol with Determiner TC555 (Kyowa Medex Co., Ltd., Tokyo, Japan); serum and liver triacylglycerol with Triglyceride G-test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Statistical analysis

Results are expressed as means with SEM. The statistical significant differences was evaluated by Tukey's test. The significance levels quoted are two-sided. Results were considered significant at $P<0.05$.

[Results]

The results are shown in Figures 1 and 2 .

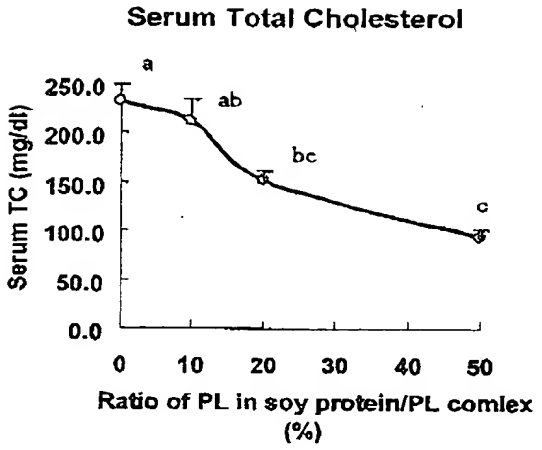

Fig.1 Serum Total Cholesterol

Values are mean±SE (n=6)

Different superscript letters in each point mean a significant difference at $p<0.05$ by tukey's test .

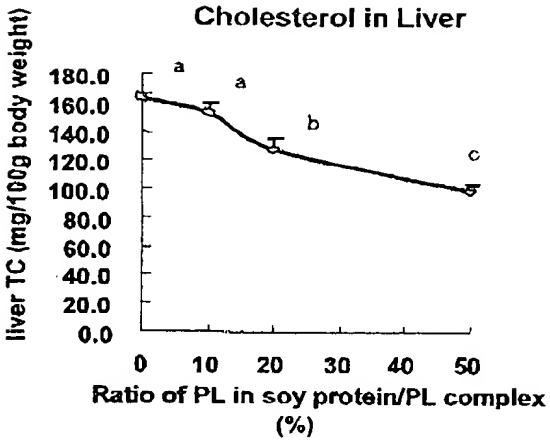

Fig.2 Cholesterol in Liver

Values are mean±SE (n=6)

Different superscript letters in each point mean a significant difference at $p<0.05$ by tukey's test .

[Conclusion]

As shown in figures 1 and 2, contents of serum total cholesterol and cholesterol in the liver were decreased depending on the ratio of phospholipid in soy protein / phospholipid complex. Especially there was significantly difference in contents of serum and liver cholesterol among ISP group, ISP-20%PL and -50%PL groups.

On the other hand, there were no significant differences in body weight gain and food intake among the groups.

Experiment II

[Materials and methods]

Preparation of soy protein /phospholipid complex

Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was dispersed in water and then stirred at 10,000 rpm for 5 minutes to make a solution. The soy protein/phospholipid complex (ISP-20%PL) was prepared by the same method as

described in Experiment I.

Animals and diets

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) weighing about 110g were used. Room temperature was maintained at 22 ± 2°C with a 12 hour cycle of light (7:00-19:00) and darkness. All the rats were housed individually in metal cages and were allowed free access to diets and water. After acclimation to a commercial stock diet (CE-2; Japan CLEA, Tokyo) for 7days, rats were divided into 4 groups of 6 rats on the basis of body weight. The rats of each group were given the diet comprising ISP, PL, ISP-20%PL or ISP+20%PL, respectively, as shown in Table 2 for 9 days. The composition of each diet was based on the American Institute of Nutrition (1977) formulation (AIN-76 diet) .

Table 2. Composition of the diets (g/kg)

	ISP	PL	ISP−20%PL	ISP + 20%PL
Casein	166.1	221.5	166.1	166.1
ISP	58.8			58.8
ISP−20%PL			73.5	
Mineral mixture*	35	35	35	35
Vitamin mixture*	10	10	10	10
Choline chloride	2	2	2	2
Cholesterol	5	5	5	5
Sodium cholate	2.5	2.5	2.5	2.5
Phospholipid		14.7		14.7
Lard	50	50	50	50
Corn oil	10	10	10	10
Sucrose	200	200	200	200
Cellulose	50	50	50	50
Starch	410.6	396.4	395.9	393

ISP, soyprotein ; PL, phospholipid ; ISP−20%PL, soyprotein with bound 20% phospholipids ; ISP + 20%PL, mixture of soyprotein and phospholipids in which phospholipids content corresponds to that of ISP−20%PL
* AIN 76 diet (American Institute of Nutrition, 1977)

Each rat was weighed its body weight and intake each 3days throughout this experiment with an electronic balance. At the end of this experiment ,after

fasting for 18 hours, the rats were anesthetized with pentobarbital (50mg/kg B.W.) Blood was sampled from abdominal artery of each rat. Whole liver was excised from each rat body. Each liver was weighed with an electronic balance after rinsed with ice-cold saline.

Lipid analysis

Serum was taken from blood with a centrifuge. Liver lipids were extracted with chloroform-methanol (2:1, v/v) in accordance with the Folch partition method, and total lipids were determined gravimetrically.

Lipid contents were determined by use of commercially available kits as follows: serum and liver cholesterol with Determiner TC555 (Kyowa Medex Co., Ltd., Tokyo, Japan); serum and liver triacylglycerol with TriglycerideG-test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan) .

Statistical analysis

Results are expressed as means with SEM. The statistical significance of differences was evaluated by Tukey's test. The significance levels quoted are two-sided . Results were considered significant at $P<0.05$.

[Results]

The results are shown in Figures 3 and 4.

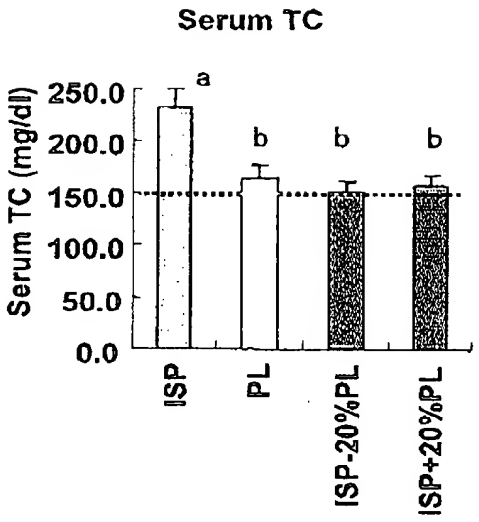

Fig.3 Serum Total Cholesterol
Values are mean±SE (n=6)
Different superscript letters in each column mean a significant difference at $p<0.05$ by tukey's test

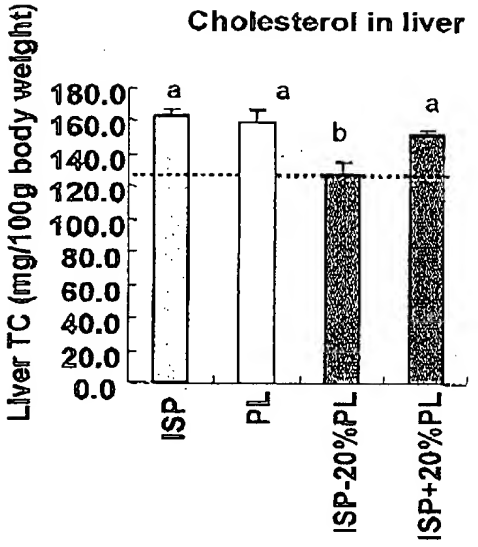

Fig.4 Cholesterol in liver
Values are mean±SE (n=6)
Different superscript letters in each column mean a significant difference at $p<0.05$ by tukey's test

[Conclusion]

As shown in Figure 3, contents of serum total cholesterol decreased in the 3 groups which fed with the diets containing phospholipids, above all, ISP-20%PL, but there were not significantly difference among these groups. As shown in Figure 4, there were significant differences in contents of liver cholesterol between ISP-20% fed group and the other groups fed with the diets containing phospholipids. These results indicated that binding soy protein with phospholipids was effective on reducing cholesterol level.

On the other hand, there were no significant differences in body weight gain and food intake among the groups.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: This 25th day of November, 2002.

Miho Takada

Miho Takada